Prolactin activation of mammary nitric oxide synthase: molecular mechanisms

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Abstract

Prolactin (PRL) is capable of stimulating both calcium and nitric oxide (NO) accumulation in mammary epithelial cells within 15 min. A calcium ionophore was also able to stimulate NO levels to an extent similar to that generated by PRL. Furthermore, maximal concentrations of PRL and the ionophore were not additive, suggesting that they were both using the same pathway, i.e. calcium. Finally, the depletion of intracellular calcium completely abrogated the effect of PRL on NO production. No other pathway known to affect NO synthase (NOS) influenced the action of PRL. Specifically, manipulations of protein phosphatase 2B, protein kinase B (PKB), protein kinase C (PKC), and arginine transport did not alter the activation of NOS by PRL. Therefore, the ability of PRL to stimulate NO production at 15 min can be completely explained by its ability to elevate intracellular calcium.

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Introduction

In mouse mammary epithelial cells, prolactin (PRL) transiently elevates nitric oxide (NO), which enhances DNA synthesis and recruits PRL receptors from internal membrane pools to the cell surface (Bolander 2001). The purpose of this study is to determine the molecular mechanisms by which PRL activates the NO synthase (NOS).

Acutely, NOS is activated by elevated calcium levels both directly via calmodulin and indirectly via stimulation of protein phosphatase 2B, which dephosphorylates the enzyme at inhibitory sites. Protein kinase B (PKB) can stimulate NOS by phosphorylating it at alternate sites (Dimmeler *et al.* 1999, Fulton *et al.* 1999). Finally, in most cells arginine, the substrate for NOS, is limiting and NOS can be activated by increasing arginine uptake (Dodd *et al.* 2000). Prolactin has the potential to affect all of these pathways (Fig. 1): it can elevate calcium levels (Bolander 1985) and stimulate both arginine transport (Rillema *et al.* 1992) and PKB activity (Al-Sakkaf *et al.* 2000, Tessier *et al.* 2001).

Materials and methods

Materials

Ovine PRL (oPRL-19) was kindly provided by the National Hormone and Pituitary Program, NIDDK (Bethesda, MD, USA), and verapamil was a gift from Knoll Pharmaceutical Co. (Whippany, NJ, USA). Hepes, lysine, glutamine, ethyleneglycol-bis-(β -amino-ethylether)-N,N'-tetraacetic acid (EGTA), phorbol 12-myristate 13-acetate (TPA) and BSA were purchased from Sigma Chemical Company (St Louis, MO, USA). A23187, calphostin C, KN-62, and wortmannin were obtained from Alexis Corporation (San Diego, CA, USA); and cyclosporin A was from Calbiochem–Novabiochem Corporation (La Jolla, CA, USA). Phosphate-buffered saline (PBS), Medium 199 with Earle's salts, and Minimum Essential Media (MEM) Select-Amine Kit were purchased from Life Technologies (Rockville, MD, USA). Collagenase, type I (179 U/mg) was obtained from Worthington Biochemicals (Freehold, NJ, USA); and the Nitric Oxide Assay Kit was from AnaBioKit^{Ready2US}, Inc. (Montreal, Canada). ⁴⁵Ca-labeled Cl₂ (30.8 mCi/mg calcium) was purchased from ICN (Irvine,

Figure 1 A scheme illustrating the possible mechanisms by which PRL could activate NO synthase and the location where various compounds used in this study act. DAG, diacylglycerol; PDK1, phosphatidylinositol-dependent protein kinase 1; PI(3)P_n, phosphatidylinositol 3'-phosphate; PLCγ, phospholipase Cγ; PP2B, protein phosphatase 2B; TPA, a phorbol ester.

CA, USA), and [methoxy-³H]inulin (452·1 mCi/g) were obtained from New England Nuclear Corp. (Boston, MA, USA).

Cell culture

Virgin mice (C3H/HeN) were obtained from the Frederick Cancer Research Facility (Frederick, MD, USA). The mice were killed by cervical dislocation and an epithelial cell-enriched fraction was isolated from mammary glands, as previously described (Vonderhaar *et al.* 1973). Cells were cultured at 37 °C in Medium 199 containing 20 mM Hepes (pH 7·6) and combinations of the following reagents, as required by the individual experiment: A23187 (32·5 nM) and EGTA (3 mM) for calcium depletion, calphostin C (100 nM), cyclosporin A (100 nM), KN-62 (10 μM), verapamil (30 μM), and wortmannin (100 nM). After 15 min, PRL (100 ng/ml), A23187 (without EGTA) for calcium elevation (32·5 nM) and/or TPA (1 ng/ml) were added to the appropriate cultures and the cells incubated for an additional 15 min. Cultures with TPA also contained BSA (4 mg/ml) to prevent the adsorption of TPA to vessel surfaces (Martel *et al.* 1983). The optimal concentrations of PRL, A23187, TPA, and verapamil were determined by dose-response curves (Bolander 1985, 2001, Caulfield & Bolander

1986). The 15-min incubation was chosen as the optimal sampling point from a previously published time course (Bolander 2001). Protein kinase inhibitors were used at concentrations that were 5–10 times their IC₅₀ (Davies *et al.* 2000).

Experiments designed to test whether arginine transport was important for early NOS stimulation either used Medium 199 containing 20 mM Hepes (pH 7·6), glutamine (5 mM), and lysine (5 mM) or used MEM containing 20 mM Hepes (pH 7·6) but without arginine. As above, cells were pre-incubated for 15 min before PRL was added.

Calcium accumulation

Since it has been shown that the initial elevation of calcium evoked by PRL arises from the influx of calcium (Alfonso *et al.* 2001), calcium accumulation was determined by measuring the uptake of ⁴⁵Ca from the medium. Mammary explants were cultured in 3 ml medium containing ⁴⁵Ca (10 μCi/ml) and [³H]inulin (5 μCi/ml). After 15 min, the explants were weighed, washed twice with 3 ml of medium without hormones followed by two washes with PBS. The tissue was dissolved in 0·6 ml of a tissue solubilizer and counted in a scintillation cocktail. [³H]insulin was used to correct for ⁴⁵Ca trapped in the extracellular space.

NO assay

Cytoplasmic NO was measured using the Nitric Oxide Assay Kit, which estimates NO from the concentrations of nitrate and nitrite, using the Griess reagent. NO production was expressed as a function of cell protein, as determined by the method of Lowry *et al.* (1951) using BSA as standard. Each dose–response curve required cells isolated from the glands of 15 mice.

Experimental rationale

In order to examine how PRL might stimulate NO production, one must first understand the mechanisms regulating NOS. As such, a brief description of the pathway being examined and the rationale for the experimental strategy are presented before the results.

Calcium

The most universal stimulator of NOS is calcium, which can act at two sites: first, it can bind calmodulin within the NOS complex to activate the enzyme directly; and second, it can stimulate protein phosphatase 2B, which dephosphorylates NOS at several inhibitory sites (Stuehr 1999). However, there are two confounding side effects: calcium also activates calmodulin-dependent protein kinase II (CaMKII), which phosphorylates and inhibits NOS (Nakane *et al.* 1991); this is thought to represent feedback inhibition. Calcium can also stimulate protein kinase C (PKC), which likewise phosphorylates NOS. However, the effects of PKC are controversial, with one group reporting NOS stimulation (Nakane *et al.* 1991) and others, inhibition (Bredt *et al.* 1992, Fleming *et al.* 2001). These potential side effects can be minimized by specific inhibitors. KN-62 is an extremely specific inhibitor of CaMKII: 10 μ M will completely inhibit CaMKII without significantly affecting any other kinase (Davies *et al.* 2000).

PKC is more problematic since there are multiple isoforms, and there are few isozyme-specific inhibitors (Hofmann 1997). However, in rats and mice, the major isoforms in the mammary gland are restricted to PKC α and PKC ζ (Connor & Clegg 1993, Birkenfeld *et al.* 1996, Masso-Welch *et al.* 1999), and PKC ζ is not activated by calcium. This simplifies the problem and allows the use of a general PKC inhibitor, such as calphostin C,

without having to determine which isoform is being affected.

The calcium ionophore, A23187, will elevate intracellular calcium levels, which should activate NOS; the use of KN-62 and calphostin C should minimize any indirect side effects of the calcium. In contrast, the use of EGTA in combination with A23187 will leach calcium from intracellular stores and sequester it in the external medium; this should suppress the ability of PRL to activate NOS. A scheme displaying the sites of action of these compounds is shown in Fig. 1.

Arginine

NOS can also be regulated by arginine concentrations, which are limiting in many cells. As such, elevated arginine levels can drive NOS activity (Dodd *et al.* 2000, Böger & Bode-Böger 2001). This regulation is facilitated by a physical association between the enzyme and the amino acid transporter, which results in substrate channeling to the enzyme (McDonald *et al.* 1997). Furthermore, PRL's inhibition of apoptosis in Nb2 cells is due, in part, to arginine-stimulated NO production, suggesting that this is a physiological mechanism of PRL action in some cells (Dodd *et al.* 2000).

Arginine uptake can be prevented by using arginine-deficient medium or by using competitive inhibitors of the transporter. The transporter used by arginine in the mammary gland is controversial; both the y^+ and the b^{0+} systems have been implicated (see the critical discussion in Shennan & Peaker 2000). As such, both 5 mM lysine and 5 mM glutamine were used to block the y^+ and b^{0+} systems respectively (Escobales *et al.* 2000). It should be noted that there are other ways that cytokines can elevate intracellular arginine concentrations, e.g. by the induction of the genes for arginine synthetase and argininosuccinate lyase and repression of the gene for arginase (Mori & Gotoh 2000). However, because PRL stimulation of NO production is rapid and brief, the incubation period will only be 15 min, thereby eliminating gene induction/repression as a mechanism for regulating cellular arginine levels.

Other potential mediators

Finally, it has been shown that PKB can phosphorylate and activate NOS (Dimmeler *et al.*

Figure 2 The effect of PRL on calcium accumulation in mammary explants. The tissue was incubated with ^{45}Ca , $[^3\text{H}]\text{inulin}$, and either the calcium ionophore, A23187 (32.5 nM), or PRL (100 ng/ml) with or without the calcium channel blocker, verapamil (30 μM). After 15 min, the explants were repeatedly washed, solubilized, and counted. Means $\pm$ S.E.s are given for six determinations.

1999, Fulton *et al.* 1999) and that this is the physiological mechanism by which some hormones stimulate NO production (Radisavljevic *et al.* 2000). If PRL activates NOS via PKB phosphorylation, its effect should be blocked by wortmannin. Wortmannin (1 μM) inhibits only phosphatidylinositol 3'-kinase (PI3K), an upstream activator of PKB, and skeletal myosin light chain kinase (Davies *et al.* 2000); the latter is not found in mammary epithelium. Furthermore, the use of wortmannin to inhibit PI3K has been validated in mammary epithelium (Bolander 1998).

Results

Figure 2 confirms previous work from this laboratory showing that PRL can rapidly elevate calcium levels in mammary tissue (Bolander 1985). The ability of verapamil to block partially this effect suggests that the calcium is primarily coming from the extracellular medium. Finally, this experiment validates the use of A23187 to raise calcium levels in mammary epithelium.

Figure 3 The effect of intracellular calcium elevation or reduction on the ability of PRL to stimulate NO production in mammary epithelial cells. Cells were depleted of intracellular calcium during a 15-min pre-incubation with the calcium ionophore, A23187 (32.5 nM), and the calcium chelator, EGTA (3 mM). All cultures also contained the CaMKII and PKC inhibitors, KN-62 (10 μM) and calphostin C (100 nM), respectively, during this pre-incubation. NO production was then determined 15 min after the addition of PRL (100 ng/ml) and/or A23187 and was estimated from the intracellular nitrite and nitrate levels. Means $\pm$ S.E.s are given for six determinations.

Figure 3 reveals that the elevation of intracellular calcium by the calcium ionophore alone is sufficient to stimulate NO production to a level comparable to that generated by PRL. Furthermore, if A23187 and PRL are using the same mechanism (i.e. calcium), maximal concentrations of each should not be additive; and indeed, this is the case. Finally, the depletion of intracellular calcium through the use of A23187 and EGTA completely prevents the action of PRL.

Figure 4 shows that neither arginine-deficient medium nor inhibitors of the arginine transporters had any effect on the ability of PRL to stimulate NO production at 15 min. Therefore, it would appear that this amino acid is not limiting in the early stages of PRL-stimulated NO production in mammary epithelium.

Figure 4 The effect of extracellular arginine (Arg) on the ability of PRL to stimulate NO production in mammary epithelial cells. Cells were pre-incubated for 15 min in either arginine-deficient medium or with the arginine transport inhibitors, lysine (Lys, 5 mM) and glutamine (Gln, 5 mM). NO production was then determined 15 min after the addition of PRL (100 ng/ml) and was estimated from the intracellular nitrite and nitrate levels. Means $\pm$ S.E.S are given for six determinations.

In addition to stimulating NOS directly, calcium can also activate protein phosphatase 2B, which can act indirectly by dephosphorylating NOS at several inhibitory sites. However, cyclosporin A, an inhibitor of protein phosphatase 2B, had no effect on the acute production of NO by PRL in mammary epithelial cells (Fig. 5). In addition, calcium can stimulate PKC, which may affect NOS positively or negatively, depending upon the system. Figure 3 demonstrated that PKC inhibition did not alter the action of PRL on NO production. In order to test the effects of PKC activation, TPA was used to stimulate this kinase. It should be noted that prolonged exposure to TPA can actually down-regulate PKC; but this was not a problem in these experiments, since the incubation period was only 15 min. Figure 5 shows that PKC activation also had no effect on the action of PRL on NOS.

Figure 5 The role of protein phosphatase 2B, PKC and the PI3K pathway in the PRL stimulation of NO production in mammary epithelial cells. Cells were pre-incubated for 15 min with a protein phosphatase 2B inhibitor (cyclosporin A, 100 nM), a PKC activator (TPA, 1 ng/ml), or a PI3K inhibitor (wortmannin, 100 nM). NO production was then determined 15 min after the addition of PRL (100 ng/ml) and was estimated from the intracellular nitrite and nitrate levels. Means $\pm$ S.E.S are given for six determinations.

Finally, NOS can be stimulated by PKB phosphorylation. However, inhibition of PI3K, which produces an activator of PKB, did not influence the ability of PRL to stimulate NO production at 15 min (Fig. 5).

Discussion

NO, at low concentrations, is an important second messenger; but at high concentrations, it can be toxic. As such, NOS is tightly controlled by multiple regulators, including calcium, phosphorylation, substrate availability, and gene transcription. It is clear from the data in this study that the acute stimulation of NOS by PRL is due to the elevation of intracellular calcium which binds to the calmodulin in the NOS complex. This finding agrees well with a report that the PRL-mediated

elevation of NO in mouse macrophages is also calcium-dependent (Kumar *et al.* 1997). Although the ability of PRL to raise calcium levels in mammary epithelium (Bolander 1985), as well as other tissues (Murphy *et al.* 1988, Villalba *et al.* 1991, Ratovondrahona *et al.* 1998), is well documented, the molecular mechanism responsible for this effect is still unknown. One likely possibility is that the calcium is released by inositol trisphosphate, whose receptor is a calcium channel; this second messenger is known to be elevated by PRL in mammary tissue (Etindi & Rillema 1988). The inositol trisphosphate, in turn, could be generated by phospholipase C γ activated via Jak-induced tyrosine phosphorylation (Hennighausen *et al.* 1997, Hynes *et al.* 1997, Bole-Feysot *et al.* 1998).

The results from this study do not eliminate the possibility that the other potential regulators examined in these experiments may be important at later time periods. For example, protein phosphatase 2B reverses the effect of CaMKII, whose phosphorylation of NOS is believed to represent negative feedback. As such, it is not likely to have a major impact on NOS activity in quiescent mammary epithelium isolated from virgin mice. However, it may acquire added significance in cells that have already been stimulated. Likewise, intracellular arginine levels appear adequate for the brief, rapid accumulation of NO triggered by PRL; but any subsequent peaks may require the replenishment of intracellular arginine pools, especially since PRL also stimulates polyamine synthesis, which would compete with NOS for this amino acid (Oka *et al.* 1978). Finally, prolonged or repeated stimulation of this enzyme would probably also affect the transcription of the NOS gene in mammary epithelium.

Prolactin is a member of the cytokine family whose classic output involves the Jak/Stat pathway. However, PRL can also couple to other soluble tyrosine kinases, such as Fyn, Src and Tec (Clevenger & Medaglia 1994, Berlanga *et al.* 1995, Kline *et al.* 2001), to the MAP kinase pathway (Das & Vonderhaar 1996), the PI3K pathway (Al-Sakkaf *et al.* 2000, Tessier *et al.* 2001), and Rac1 (Kline *et al.* 2001). Furthermore, the PRL receptor can even directly bind 2',5'-oligoadenylate synthetase (McAveney *et al.* 2000) and 17 α -hydroxysteroid dehydrogenase/17-ketosteroid reductase (Nokelainen *et al.* 1998). This laboratory has previously reported

that NO is another mediator of PRL action in mammary epithelium (Bolander 2001), and this study has delineated the molecular mechanism by which PRL activates the NOS, namely that PRL acutely stimulates NOS by elevating calcium levels.

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